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(71) Applicant: CHILDRENS HOSPITAL OF LOS ANGELES [US/US]; 4650 Sunset Boulevard, Los Angeles, CA 90027 (US).

(72) Inventors: HALL, Frederick, L. ; CARBONARO-HALL, Denise ; 3899 Wawona Street, Los Angeles, CA 90065 (US).

(74) Agent: MISROCK, S., Leslie; Pennie & Edmonds, 1155 Avenue of Americas, New York, NY 10036 (US).

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(54) Title: MONOSPECIFIC ANTIBODIES AND ANTISERA TO THE REGULATORY SUBUNIT OF PROLINE-DIRECTED PROTEIN KINASE

(57) Abstract

The present invention provides for monospecific antibody directed toward human cyclin A, the regulatory subunit of proline-directed protein kinase (PDPK). It is based, in part, on the discovery that antibody directed toward defined regions of human cyclin A was highly specific for PDPK, and bound selectively to actively dividing cells in human tumors, but not to quiescent cells. Accordingly, the antibodies of the invention may be extremely useful in the diagnosis and grading of cancer and other proliferative diseases, and may also be used therapeutically as targeting or directly cytotoxic agents in diseases associated with aberrant control of cell division.

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MONOSPECIFIC ANTIBODIES AND ANTISERA TO THE REGULATORY SUBUNIT OF PROLINE-DIRECTED PROTEIN KINASE

1. INTRODUCTION

The present invention provides for monospecific antibody directed toward the regulatory subunit of proline-directed protein kinase (PDPK). Such antibody may be used in the diagnosis and/or treatment of diseases that manifest a disorder of PDPK expression.

2. BACKGROUND OF THE INVENTION

2. 1. CYTOPLASMIC SERINE/THREONINE PROTEIN KINASES IN MOLECULAR SIGNALLING

The executive role of various growth factors, growth factor receptors, and/or tyrosine kinases in the control of mammalian cell proliferation is now well established, as is the potential for viral subversion and/or oncogenic disturbance in each of these components. However, until recently, the actual mechanisms of signal transduction, signal amplification, and the biochemical pathways stimulated by the mitogenic growth factors and oncogenic tyrosine kinases remained largely unresolved. Several lines of evidence suggest that the activation of cytosolic serine/threonine protein kinases, directly or indirectly, by tyrosine activity, may provide a crucial function downstream from membrane-mediated events in molecular signalling. The following diagram (Figure 1) depicts two hypothetical pathways for the cascade of protein phosphorylation events that are governed by polypeptide growth factors: one pathway invokes the activity of intracellular second messengers (or mediators) while the other typifies a more direct cascade of protein phosphorylation events.

The first functional links between growth factor-stimulated tyrosine kinase activity and the classical enzymology of the second messengers, most notably PI kinase and phospholipase c, have been reviewed (see Witters, L., Curr. Opin. Cell Biol., 1990, 2:212-220; Cooper, J., Curr. Opin. Cell Biol. 1990, 2:285-295). The second pathway, i.e., the direct transduction of tyrosine kinase activity to serine/threonine kinase activity, provides the conceptual basis for understanding a relatively new and potentially important enzymology.

There is increasing evidence that a number of cytosolic serine/threonine kinases are activated directly by tyrosine phosphorylation. The prominent 42 kDA tyrosine kinase substrate phosphorylated in mammalian cells that have been stimulated by EGF, PDGF, IGF-II, or TPA has been identified as a cytosolic serine/threonine protein kinase that is referred to as MAP kinase (the acronym MAP purportedly stands for both "mitogen-activated protein" and "microtubule-associated protein" (Rossomando, et al., Proc. Natl. Acad. Sci. U.S.A., 1989, 86:6940-6943; Boulton, et al., Science, 1990, 249:6940-6943).

Other studies have demonstrated that Raf-1, a cytoplasmic oncogenic serine/threonine kinase, which is stimulated by several mitogenic growth factors, is directly activated by tyrosine phosphorylation resulting directly from the intrinsic kinase activity of the PDGF receptor (Morrison, et al., 1989, Cell 58:649-657). Raf-1 is also activated directly by serine phosphorylation (Kovacina, et al., 1990, J. Biol. Chem. 265:12115-12118). Once activated by covalent modification (or mutation), the Raf-1 kinase apparently phosphorylates a factor (or factors) which stimulate the transcription of c-fos and B-actin

(Jamal, S. and Ziff, E., 1990, Nature 344:463-466), linking this serine/threonine kinase to the induction of early response genes. These findings indicate that cytosolic serine/threonine protein kinases may be among the more important targets for cellular as well as viral tyrosine kinases. Whether the initial mechanism of signal transduction is carried out directly via tyrosine phosphorylation or indirectly via the generation of second messengers, it is clear that the activation of specific serine/threonine protein kinases is a fundamental component of mitogenic and/or oncogenic signalling pathways.

2. 2. THE EXECUTIVE PROTEIN KINASE(S) OF S PHASE

In contrast to the abbreviated cell cycles of oocytes and early stage embryos (Murry, A. and Kirschner, M., 1989, Nature 339:275-280; Murry, A. and Kirschner, M., 1989, Science 246:614-621), a major point of control in the mammalian somatic cell cycle is located at the G1/S phase transition. This G1 restriction point in the somatic cell cycle is analogous to START in the yeast cell cycle, where external factors come into play. It is during G1 that serum factors such as PDGF, EGF and IGF-1 act in a concerted manner to stimulate the transition to S phase (Gould, K. and Nurse, P., 1989, Nature 342:39-45), and after which exogenous growth factors are no longer required to complete the cell division cycle. One of the major challenges of modern regulatory biology is to identify the executive growth factor-sensitive protein kinases that govern the initial, instructive phases (i.e., G0 and G1) of the cell cycle. Out of all the identified protein kinases, one particular serine/threonine kinase stands out as the critical sentinel of S phase. It is here at START

that $p34^{cdc2}$ is predicted from genetic studies in yeast to serve a vital function (reviewed in Nurse, P., Nature 1990, 334:503-508), in addition to its executive role in promoting M phase. More recent studies utilizing specific immunodepletion (Blow, J. and Nurse, P., 1990, Cell 62:855-862) and antisense oligodeoxynucleotide strategies (Furukawa, et al., 1990, Science 250:805-808) have confirmed that $p34^{cdc2}$ plays a pivotal role in the initiation of DNA replication in higher eukaryotes.

Based on genetic, molecular, and biochemical inferences, there is considerable speculation surrounding the role of G1/S phase-specific cyclins in cell cycle control (Murry, A. and Kirschner, M., 1989, Nature 339:275-280; Murry, A. and Kirschner, M., 1989, Science 46:614-621; Cross et al., 1989, Ann. Rev. Cell Biol. 5:341-395; Pines, J. and Hunter, T., 1990, The New Biologist 2:389-401). A large number of studies in this area have led to the speculation that there exists an operationally defined S Phase Promoting Factor in somatic cells that is analogous to the well defined Maturation- or M-phase Promoting Factor of oocytes (Lewin, B., 1990, Cell 61:743-752) . Furthermore, it has been postulated that the enzymatic component of this S Phase Promoting Factor would contain $p34^{cdc2}$ in a complex with a G1-specific cyclin (Murry, A. and Kirschner, M., Nature, 1989, 339:275-280), a cyclin-like protein (Lewin, B., 1990, Cell 61:743-752; Pines, J. and Hunter, T. 1989, Cell 58:833-846), or possibly cyclin A itself (Murry, A. and Kirschner, M., 1989, Science 246:614-621). The A-type cyclin is a distinctive protein which has been previously characterized in phyolgenetically lower animals but has only recently been detected in mammals (Human cyclin A is adenovirus E1A-associated protein

p60 and behaves differently from cyclin B. Nature 1990, 346:760-763; Wang, et al., 1990, Nature 343:555-557). In Drosophila embryos, cyclin A accumulates during interphase, at which time it is found exclusively in the cytoplasm (Lehner, C. and O'Farell, P., 1989, Cell 56:957-968). Early in prophase, cyclin A translocates to the nucleus where it is thought to participate in the initiation of mitotic events which lead to chromosome condensation and nuclear envelope breakdown. Further studies demonstrated that after a specific number of embryonic cell divisions the expression of cyclin A, but not cyclin B, becomes necessary for continued cell division (Lehner, C. and O'Farell, P., 1989, Cell 61:535-547). In other words, as embryonic cells become specialized, the specific requirement for cyclin A emerges. Recently, a series of innovative *in vitro* studies of the regulation of DNA replication, utilizing partially purified mammalian cell extracts, have demonstrated not only that the $p34^{cdc2}$ kinase is necessary, but that the addition of cyclin A to a G1 cell extract is sufficient to initiate DNA replication (D'Urso, et al., 1990, Science 250:786-791).

2. 3. CHARACTERIZATION OF THE CYTOSOLIC PROLINE-DIRECTED PROTEIN KINASE

Analysis of specific phosphorylation sites in multiply phosphorylated proteins, such as glycogen synthase and histone H1, has proven to be of extraordinary utility in assessing the activities and dynamics of discrete protein kinase systems during the course of cellular activation and/or cell division. Ongoing investigations of the site-specific phosphorylation of tyrosine hydroxylase in rat pheochromocytoma led to the discovery of the cytosolic proline-directed protein kinase (PDPK) (Vulliet, et

al., 1989, J. Biol. Chem. 264:16293-16298), and have provided biochemical evidence linking the activity of this cytosolic serine/threonine kinase system to the biochemical signalling cascades activated by growth factors (see Figure 2). Further studies of phosphorylation site-specificity revealed that the PDPK will phosphorylate a particular peptide or protein if and only if the phosphoacceptor site is flanked by an adjacent carboxyl-terminal proline residue, indicating a minimal recognition site specificity of -X-Ser/Thr-Pro-X- (Vulliet, et al., 1989, J. Biol. Chem 264:16293-16298; Hall, et al., 1990, J. Biol. Chem. 265:6944-6948; also see Kemp, B. and Pearson, R., 1990, TIBS 15:342-346). Other studies have demonstrated that this minimal concensus sequence is not only selective for PDPK, but appears to be phosphorylated, under certain circumstances, by the "growth-associated", M phase-specific H1K (Pines, J. and Hunter, T., 1990, The New Biologist 2:389-401; Kemp, B. and Pearson, R., 1990, TIBS 15:342-346; Moreno, S. and Nurse, P., 1990, Cell 61:549-551). While this striking similarity of phosphorylation site specificity is counterposed by major differences in subcellular localization, physiological activation, chromatographic behavior, and physicochemical properties; the molecular nature of PDPK and, hence, the biochemical basis of this curious overlap in substrate specificity, remained a mystery (Pines, J. and Hunter, T., 1990, The New Biologist 2:389-401).

During this past year, we have succeeded in purifying PDPK to near homogeneity from the cytosol of mouse FM3A mammary carcinoma cells, as well as from human Wilms' tumors, and have identified the unique subunit configuration of PDPK (Hall et al., 1990, J. Cell Biol. 111:341a, Hall et al., 1991, J. Biol. Chem.

266:17430-17440; Hall and Vulliet, 1991, Current Opin. Cell. Biol. 3:176-184). The dominant 58 kDA phosphoprotein present in these preparations has been identified as a mammalian A-type cyclin.

Recent studies of E1A-associated proteins in adenovirus-infected cells led to the identification of a novel $p34^{cdc2}$-containing protein kinase complex that is also distinct from the M phase-specific H1K (Giordano, et al., 1989, Cell 58:981-990). In this E1A-associated kinase, $p34^{cdc2}$ is complexed with a regulatory subunit (p60) that is clearly distinguishable from $p62^{cyclin\ B}$ and has recently been identified as a mammalian A-type cyclin (Pines, J. and Hunter, T., Nature, 1990, 346:760-763). Remarkably, it was the association with the E1A viral protein, rather than the specific probes for H1K (G6 antisera and/or $p13^{suc1}$-Sepharose beads), that led to the independent discovery of $p34^{cdc2}/p58(60)^{cyclin\ A}$ PDPK. Studies of the $p34^{cdc2}/p58^{cyclin\ A}$ kinase complex have confirmed that this enzyme is clearly active during interphase (Giordano, et al., 1989, Cell 58:981-990) and that this activity increases progressively during S phase (Pines, J. and Hunter, T., Nature 1990, 346:760-763), in parallel with the phosphorylation of both the p53 (Bischoff et al., 1990, Proc. Natl. Acad. Sci. U.S.A. 87:4766-4770) and the Rb (see Pines, J. and Hunter, T., 1990, The New Biologist 2:389-401) tumor suppressor proteins. Furthermore, potentially important differences in the substrate preferences (Minshull et al., 1990, EMBO J. 9:2865-2875) as well as in the temporal pattern of cyclin A (versus cyclin B) expression have recently been confirmed (Human cyclin A is adenovirus E1A-associated protein p60 and behaves differently from cyclin B. Nature 1990, 346:760-763; Minshull et al., 1990, EMBO J. 9:2865-

2875). In view of these recent developments, it is now safe to assume that $p34^{cdc2}/p58^{cyclin\ B}$ PDPK and $p34^{cdc2}/p62^{cyclinB}$ H1K perform separate functions in mammalian somatic cells, and that additional differences in the biochemical regulation and physiological function of these distinctive kinase complexes will be forthcoming.

2. 4. CYCLIN A IN NORMAL AND NEOPLASTIC CELL GROWTH

In invertebrate oocytes and early embryos, cyclin proteins (as the name implies) exhibit cell cycle-dependent oscillations in synthesis and destruction; hence, the protein synthesis requirement for each successive cell cycle may be partly explained by a requirement for cyclin proteins (Murry, A. and Kirschner, M., Nature, 1989, 339:275-280; Murry, A. and Kirschner, M., Science 1989, 246:614-621; Minshull, et al., 1989, J. Cell Sci. Suppl. 12:77-97). In invertebrate oocytes and early embryos, in which a well-defined G1/S restriction point is absent, cyclin A and cyclin B appear to perform similar if not overlapping functions; however, as embryonic cells become more specialized, the specific requirement for cyclin A emerges and dramatic differences in the levels of cyclin A between germinal and somatic cells can be detected ((Lehner, C. and O'Farell, P., 1989, Cell 56:957-968; (Lehner, C. and O'Farell, P., 1989, Cell 61:535-547). In Drosophila embryos the behavior of cyclin A is particularly interesting in that only a certain fraction of (maternally-provided) cyclin A is destroyed with each successive cell cycle ((Lehner, C. and O'Farell, P., 1989, Cell 56:957-968), providing a bypass of this particular biosynthetic requirement. Perhaps it is only the active fraction of cyclin A which actually enters the nucleus that is targeted for

proteolytic destruction (Minshull et al., 1989, J. Cell Sci. Suppl. 12: 77-97. Anaplastic cancer cells, like early-stage embryos, bypass this particular requirement, and/or subvert the cyclin A-associated kinase (PDPK) to efficiently traverse the G1/S boundary.

Investigations into the mechanisms of viral subversion in mammalian cells have independently focussed on cyclin A and its associated protein kinase activity, providing new indications of a potential role in cellular transformation. The E1A transforming protein in adenovirus-infected cells is known to form a complex with the Rb tumor suppressor protein, which somehow leads to the disruption of its growth suppression function. The identification of cyclin A as an E1A-associated protein (Giordano, et al., 1989, Cell 58:981-990) and the demonstration that the $p34^{cdc2}/p58^{cyclin\ A}$ PDPK is enzymatically active during S phase (Pines, J. and Hunter, T., 1990, Nature 346:760-763) provides a physical as well as a temporal link to the phosphorylation of the Rb protein. This is very significant in that it has been predicted that the protein kinase that phosphorylates and inactivates the Rb tumor suppressor protein would, in principle, be the product of a proto-oncogene (DeCaprio et al., 1989, Cell 58:1085-1095). Further studies have suggested that the $p34^{cdc2}/p58(60)^{cyclin\ A}$ protein kinase is a physiologically relevant enzyme in terms of regulating p53 tumor suppressor function as well (Bischoff et al., 1990, Proc. Natl. Acad. Sci. U.S.A. 87:4766-4770). Recent studies of the hepatitis B virus have identified an intron of the cyclin A gene as a specific target site for viral integration, directly implicating the cyclin A system in the pathogenesis of human hepatocellular carcinoma (Wang,

et al., 1990, Nature 343:555-557). In addition, this study provides preliminary indications that the induction of cyclin A transcription may be an initial event associated with the transformation of these particular cells (Wang, et al., 1990, Nature 343:555-557). Thus, it appears that at least two transforming viruses, in their ultimate efficiency, have targeted cyclin A as a pivotal and instructive locus of action, identifying the regulatory subunit of PDPK, rather than the ubiquitous catalytic subunit, as the product of a candidate proto-oncogene.

3. SUMMARY OF THE INVENTION

The present invention provides for monospecific antibody directed toward human cyclin A, the regulatory subunit of proline-directed protein kinase (PDPK). It is based, in part, on the discovery that antibody directed toward defined regions of human cyclin A was highly specific for PDPK, and bound selectively to actively dividing cells in human tumors, but not to quiescent cells. Accordingly, the antibodies of the invention may be extremely useful in the diagnosis and grading of cancer and other proliferative diseases, and may also be used therapeutically as targeting or directly cytotoxic agents in diseases associated with aberrant control of cell division. In a particular embodiment, the present invention provides for monoclonal antibody CHLA-3.

4. DESCRIPTION OF THE FIGURES

Figure 1: Presiding near the headwaters of the major stimulus-response cascades, cytosolic serine/threonine kinases and their respective patterns of protein phosphorylation form a functional link

between membrane-mediated events and the subsequent alternations in cellular physiology. This diagram depicts two distinctive modes of serine/threonine kinase activation: one involves the classical rise in second messengers or mediators, while the other typifies a more direct cascade of protein phosphorylation events.

Figure 2: Stimulation of PDPK activity by growth factors, growth factor receptors, and tyrosine kinase activity. Subconfluent rat PC12 pheochromocytoma cells were treated with NGF (50 ng/ml, 1 min); human A431 epidermoid carcinoma cells were treated with EGF (5 ng/ml, 1-2 min), mouse HIH 3T3 cells were transfected with the HER2 receptor (constitutive activity during log phase growth); purified PDPK was incubated in the presence or absence of purified $pp60^{c\text{-}src}$. PDPK activity was assessed by the phosphorylation of the synthetic peptide $TH^{2\text{-}16}$ as described Vulliet, et al., J Biol. Chem 1989, 264:16293-16298. Each value represents the mean +/- SE of 5-9 independent determinations ($p < 0.01$).

Figure 3: A model of PDPK stimulation by receptor-mediated mechanisms. Based on the information described within the text, it is predicted that the activity of the $p34^{cdc2}/p58^{cyclin\ A}$ heterodimer is limited, not only by the availability of cyclin A subunit, but by specific covalent modification. In this model, a subset of the cyclin A pool (lightly shaded) combines with a subset of the $p34^{cdc2}$ pool (lightly shaded) to form the PDPK heterodimer. The activated PDPK heterodimer (darkly shaded) undergoes autophosphorylation. The assembly of the PDPK heterodimer and/or the activity of this kinase complex is predicted to be under the influence of growth factors.

Figure 4: Western blot showing the specific binding of CHLA-1 antiserum to both forms of cyclin A, the regulatory subunit of PDPK, showing phosphorylation of cyclin A on tyrosine residues. Note: the comigration of the lower band of the cyclin A doublet with $pp60^{c\text{-}src}$ precluded the assessment of its possible phosphorylation on tyrosine residues under these conditions. Lane 1, PDPK only; lane 2, PDPK plus $pp60^{c\text{-}src}$, Lane 3, $pp60^{c\text{-}src}$ only.

Figure 5: Western blots of various preparations of anti-PDPK antibodies, showing specific binding to $p58^{cyclin\ A}$. Immunogens were: A. P1; B. P1, P2 and P4.

Figure 6: Photomicrographs of tumor frozen sections stained with anti-cyclin A antibodies (CHLA-1; directed toward P1) and biotin-streptavitin conjugated immuno-alkaline phosphatase for visualization. Tumors are: A. pilocytic astrocytoma; B. Anaplastic astrocytoma, and C. Pigmented PNET (Primitive Neuroectodermal tumors).

Figure 7: Three regions of the Cyclin-A molecule were targeted for antibody generation; the N-terminal region, corresponding to P3 peptide, the cyclin box, corresponding to P2 peptide, and the C-terminal region, corresponding to P1 and P4 peptides. Amino acids comprised in each region are denoted by numbers above the boxed region names. Monospecific antibodies generated toward (i) P3 are termed CHLA-3 (monoclonal); (ii) P2 are termed CHLA-2 (polyclonal); and (iii) P1 and P4 are, respectively, CHLA-1 and CHLA-4 (both polyclonal). B. Western blot of recombinant protein probed with monospecific antibodies CHLA-1, CHLA-2, CHLA-3, or C160. C. Western blot of MG-63 cell lysates prepared from cells in G_0 or S phase, using monospecific antibodies CHLA-1, CHLA-3, CHLA-4, or C160 as probes. Lysate in lanes

probed by CHLA-3, CHLA-4, and C160 was prepared from S-phase MG-63 cells. D. Immunoprecipitation of MG-63 cell lysates by CHLA-1, CHLA-4, C160, anti-cdc-2 sera, or by protein-A Sepharose (control). E. Western blots of lysates prepared from Ewing's sarcoma (EW-1) cells or MG-63 (osteosarcoma cells) probed with CHLA-1.

5. DETAILED DESCRIPTION OF THE INVENTION

For purposes of clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the following subsections:

(i) antibody preparation;

(ii) diagnostic methods; and

(iii) therapeutic methods.

5. 1. ANTIBODY PREPARATION

The present invention provides for antibodies, antibody derivatives and fragments specific for defined regions and functional domains of human cyclin A, the regulatory subunit of PDPK. In particular, the present invention provides for monospecific antibodies defined herein. In preferred specific embodiments of the invention, the antibodies, or derivatives or fragments thereof, are capable of binding to one of the following peptides, which are fragments of cyclin A:

1) KYHGVSLLNPPETLNL (P1)

2) CLVEVGEEYKLQNE (P2)

3) CIHVDEAEKEAQKKPAE (P3)

4) CLMDLHQTYLKAPQHAQQSIREKYKNSKYHG (P4)

Various procedures known in the art may be used for the production of polyclonal antibodies that recognize any of these four peptides. For the production of antibody, various host animals,

including but not limited to rabbits, mice, rats, etc., can be immunized by injection with at least a portion of one of the four above-mentioned peptides (P1, P2, P3 or P4), which may be comprised in a larger molecules and/or derivatized. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (Bacile Calmette-Guerin) and, Corynebacterium parvum.

In specific embodiments of the invention, peptides P1 through P4 may be conjugated to keyhole limpet hemocyanin, suspended in PBS, mixed with Freund's adjuvant and injected into a rabbit about three times in 14 day boost/bleed intervals according to standard protocols.

For use as immunogens, the peptides P1-P4, or fragments thereof, may be produced by any method known in the art, including by chemical synthesis, purification, or by genetic engineering methods.

For preparation of monoclonal antibodies directed toward peptides P1 through P4, any technique which provides for the production of antibody molecules by continuous cell lines in culture or by bacteria may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in "Monoclonal Antibodies and Cancer

Therapy," Alan R. Liss, Inc. pp. 77-96) and the like are within the scope of the present invention.

The monoclonal antibodies for therapeutic use may be human monoclonal antibodies or chimeric human-mouse (or other species) monoclonal antibodies. Human monoclonal antibodies may be made by any of numerous techniques known in the art (e.g., Teng et al., 1983), Proc. Natl. Acad. Sci., U.S.A. 80-7308-7312; Kozbor et al., 1983, Immunology Today 4:72-79; Olsson et al., 1982, Meth. Enzymol. 92:3-16). Chimeric antibody molecules may be prepared containing a mouse antigen-binding domain with human constant regions (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851, Takeda et al., 1985, Nature 314:452).

A molecular clone of an antibody to a cyclin A epitope such as to peptides P1-P4 or fragments thereof, can be produced by known techniques. Recombinant DNA methodology (see e.g., Maniatis et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York) may be used to construct nucleic acid sequences which encode a monoclonal antibody molecule, or antigen binding region thereof.

Antibody molecules may be purified by known techniques, e.g., immunoabsorption or immunoaffinity chromatography, chromatographic methods such as HPLC (high performance liquid chromatography), or a combination thereof, etc.

The present invention provides for antibody molecules as well as fragments of such antibody molecules.

Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment which can be produced

by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

The present invention further provides for monoclonal antibody CHLA-3, which recognizes P3, and is produced by hybridoma CHLA-3-13 as deposited with the American Type Culture Collection and assigned accession number _____, and for antibodies that competitively inhibit the binding of CHLA-3 to P3, and for fragments of CHLA-3 as well as antibodies that compete with CHLA-3 for P3 binding. Competing antibodies and antibody fragments may bind to P3 with the same affinity, greater affinity, or less affinity compared to CHLA-3.

5. 2. DIAGNOSTIC METHODS

The antibodies, antibody fragments or derivatives of the invention may be used to diagnose diseases characterized by aberrant cellular proliferation. The present invention provides for a method of diagnosing a disorder of cellular proliferation in a patient comprising (i) exposing a sample obtained from the patient to monospecific anti-cyclin A antibody under conditions that permit antibody binding; (ii) detecting and measuring anti-cyclin A antibody binding; and (iii) comparing the amount of antibody binding to the sample with the amount of monospecific anti-cyclin A antibody binding to a comparable sample from a normal person, such that a significant difference between the amount of antibody bound to the patient sample and the normal sample is indicative of a disorder of cellular proliferation.

Antibody binding may be detected and measured by any method known in the art, including, but not limited to, Western blotting, immunoprecipitation, and immunocytochemical studies.

Suitable samples include, but are not limited to, portions of tissue obtained from a patient, cells obtained from a patient, or body fluid, such as blood, cerebrospinal fluid, urine, or pleural fluid obtained from a patient, to name but a few examples.

In some circumstances, it may be useful to determine the overall level of anti-cyclin A antibody binding to a sample. For example, in testing a patient for the presence of malignancy or viral infection, it may be useful to test the overall level of anti-cyclin A antibody binding to the sample as a whole.

In other examples, it may be preferable to be able to identify localized areas of anti-cyclin A antibody binding. For example, in the diagnosis and/or staging of cancer, it may be useful to identify mitotically active cells. As illustrated in Section 7, infra, anti-cyclin A antibody according to the invention may be used to selectively bind to, and thereby identify, mitotically active cells.

The present invention provides for diagnostic methods performed in vitro (i.e. the sample is outside the patient), or in vivo (i.e. the sample is inside the patient).

As a specific nonlimiting example, anti-cyclin A antibody prepared according to the invention may be used to selectively identify mitotically active cells in tumor tissue by exposing acetone-fixed tissue to anti-cyclin A antibody, and then visualizing binding using the biotin-streptavidin conjugated immuno-alkaline phosphatase method. Preferably, tissue may be

preincubated with 170 glacial acetic acid diluted in phosphate buffered saline (PBS) to block endogenous alkaline phosphatase.

The present invention may be used to diagnose and stage malignancy, including, but not limited to, neuroblastoma, astrocytoma, medullo blastoma, PNET, leukemia, lymphoma, lung cancer, etc.; myeloproliferative disease [disorders of pregnancy, e.g. hydatid form mole]; autoimmune disease, and sarcoid.

5. 3. THERAPEUTIC METHODS

The present invention also provides for methods of treating disorders of cellular proliferation comprising administering to a patient in need of such treatment, an effective amount of monospecific anti-cyclin A antibody, such that actively dividing cells bearing PDPK may be damaged and/or killed. Such antibodies may themselves be capable of eliciting a cytotoxic response (e.g. be capable of antibody dependent cytotoxicity or complement directed cytotoxicity) or may be linked to a toxic agent, including, but not limited to, a toxin, cytokine, radioisotope, anti-proliferative agent, or any chemotherapeutic agent. Many therapies currently used in the treatment of disorders of proliferation are selectively toxic to dividing cells; the antibodies and antibody derivatives and fragments of the invention provide a means for further focusing the therapeutic agent on proliferating cells, thereby achieving a higher effective dose per cell. The antibodies of the invention may be administered systemically or locally, by any technique known in the art, in a suitable pharmaceutical carrier. The same

disorders described in Section 5.2, supra, may be treated according to this section.

6. EXAMPLE: MONOSPECIFIC ANTISERA CHLA-1 RECOGNIZED BOTH SUBUNITS OF PROLINE-DIRECTED PROTEIN KINASE

PDPK was purified from the cytosol of mouse FM3A cells by a modification of the methods described in Hall, et al., J. Biol. Chem. 1990, 265:6944-6948. The dominant protein in these preparations is identified as cyclin A, as shown in the first Western blot that has been probed with a rabbit polyclonal antiserum (CHLA-1) directed against the synthetic peptide P1 (supra) corresponding to a specific region in the C-terminal domain of human cyclin A. Both bands of the $p58^{cyclin\ A}$ doublet (arrows) bound to CHLA-1 and are modified by autophosphorylation, as shown in Figure 4. Western blotting with anti-phosphotyrosine antibodies (PY20) reveal, not only that the upper band of the $p58^{cyclin\ A}$ doublet is phosphorylated on tyrosine residues in vivo (single arrow in Figure 4), but that this tyrosine phosphorylation can be increased in vitro by the addition of purified $pp60^{c\text{-}src}$.

Figure 5 further shows the binding of antisera prepared toward P1, P2, and P4 on a Western blot of PDPK preparations from human osteosarcoma cells. The binding of various bleeds of primary antisera was followed by horseradish peroxidase-conjugated secondary antibodies and developed in diaminobenzadiene to visualize the reaction products. Specific staining of the 58 kDa subunit of PDPK was generally observed after the second bleeds and was further enhanced by the purification of the corresponding IgG fractions. High titre antisera capable of recognizing exclusively this protein has subsequently been characterized.

7. EXAMPLE: SELECTIVE BINDING OF CHLA-1 TO ACTIVELY DIVIDING TUMOR CELLS

Tumors were obtained by neurosurgical resection, embedded in OCT compound, snap frozen, and stored at -70°C. Frozen sections were fixed in acetone and the antigen was detected using purified rabbit anti-cyclin A antibodies prepared toward peptide P1 (CHLA-1) and the biotin-streptavidin conjugated immuno-alkaline phosphatase method of visualization. Pre-incubation with 1% glacial acetic acid diluted in PBS was used to block the endogenous alkaline phosphatase activity in the tumor tissues. As seen in Figure 6, CHLA-1 binding indicated that the regulatory subunit of PDPK is expressed only in actively dividing tumor cells (as well as the hyperplastic endothelium of the slowly growing pilocystic astrocytoma). Selective staining of proliferative cells was also observed in the normal thymus, where the quiescent cortical cells were readily distinguished from the mitotically active cells of the germinal centers. The proportion of cells that express cyclin A in a given tumor may provide an index of proliferation that is far superior to the mitotic index.

8. EXAMPLE: CHARACTERIZATION OF MONOSPECIFIC ANTIBODIES TO HUMAN CYCLIN A

Western analysis of synchronized MG-63 cells with additional antibodies selective for human cyclin A detected a complex pattern of immunoreactive bands at 58-60 kDa. We targeted specific regions of human cyclin A (See Figure 7A), synthesized model peptides, P1, P2, P3, and P4 (See Section 5.1, supra), and generated several monospecific antibodies directed against defined epitopes of cyclin A, including polyclonal antibodies to P1, P2, and P4, termed CHLA-1, CHLA-2, and CHLA-4, respectively, and a monoclonal

antibody to P3,termed CHLA-3. These antibodies were characterized in terms of Western blotting of recombinant protein as well as immunoprecipitation. For Western blotting analysis, the same blot was cut into strips and each strip was probed with primary antibodies (CHLA-3 was applied as a hybridoma supernatant) and appropriate enzyme-conjugated secondary antibodies, followed by development of the colored reaction products (Figure 7B).

Differential staining of the cyclin A bands was observed with different monospecific antibodies. MG-63 cell lysates were prepared from quiescent (Go) and/or S phase cells (S) and analyzed by Western blotting with the specified antibodies (Figure 7C). The three lanes labeled CHLA-3, CHLA-4, and C160 represent immunoblotting of the same S phase extracts from the same Western transfer with the specified antibodies (monoclonal antibody C160 is described (monoclonal antibody C160 is described by Giordano et al., 1989, Cell 58:981-990). Note the multiple cyclin A bands recognized by CHLA-1, 3, and 4, which indicates that multiple forms of cyclin A exist in mammalian as well as insect cells. The C160 blot was purposefully overdeveloped to ensure the absence of immunoreactivity toward the more rapidly migrating forms of cyclin A.

Cyclin A was specifically immunoprecipitated from MG-63 cell lysates by CHLA-1, CHLA-4, C160 Mab, and anti-cdc2 antisera but not by the protein A-Sepharose (Control) used to collect the immune complexes (Figure 7D). The monoclonal antibody C160 was used to identify the immunoprecipitated cyclin A protein in the subsequent Western blot.

CHLA-1 specifically recognized the cyclin A protein (brackets) in crude (asynchronous) lysates

obtained from Ewings sarcoma cells (EW-1) and Osteosarcoma cells (MG-63), revealing multiple forms of this protein in both cell lines while exhibiting little if any nonspecific immunoreactivity (Figure 7E).

9. DEPOSIT OF HYBRIDOMA

Hybridoma cell line CHLA-3-13 was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, and assigned accession number _____.

Various publications have been cited herein, which are hereby incorporated by reference in their entirety.

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganisms referred to on page 22, lines 7-11 of the description '

A. IDENTIFICATION OF DEPOSIT '
Further deposits are identified on an additional sheet '

Name of depositary institution '

American Type Culture Collection

Address of depositary institution (including postal code and country) '

12301 Parklawn Drive
Rockville, MD 20852
US

Date of deposit ' April 29, 1992 Accession Number ' _____

B. ADDITIONAL INDICATIONS ' (leave blank if not applicable). This information is continued on a separate attached sheet

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE ' (If the indications are not all designated States)

D. SEPARATE FURNISHING OF INDICATIONS ' (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later ' (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the International application when filed (to be checked by the receiving Office)

_____
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau "

was

_____
(Authorized Officer)

Form PCT/RO/134 (January 1981)

WHAT IS CLAIMED IS:

1. A monospecific antibody that binds to human cyclin A, the regulatory subunit of proline-directed protein kinase.

2. The antibody of claim 1 that binds to the peptide: KYHGVSLLNPPETLNL, termed P1.

3. The antibody of claim 1 that binds to the peptide: CLVEVGEEYKLQNE, termed P2.

4. The antibody of claim 1 that binds to the peptide: CIHVDEAEKEAQKKPAE, termed P3.

5. The antibody of claim 1 that binds to the peptide: CLMDLHQTYLKAPQHAQQSIREKYKNSKYHG, termed P4.

6. The antibody of claim 1 that is a monoclonal antibody.

7. The antibody of claim 2 that is a monoclonal antibody.

8. The antibody of claim 3 that is a monoclonal antibody.

9. The antibody of claim 4 that is a monoclonal antibody.

10. The antibody of claim 5 that is a monoclonal antibody.

11. A method of diagnosing a disorder of cellular proliferation in a patient comprising (i)

exposing a sample obtained from the patient to a monospecific anti-cyclin A antibody under conditions that permit antibody binding; (ii) detecting and measuring anti-cyclin A antibody binding; and (iii) comparing the amount of antibody binding to the sample with the amount of monospecific anti-cyclin A antibody binding to a comparable sample from a normal person, such that a significant difference between the amount of antibody bound to the patient sample and the normal sample is indicative of a disorder of cellular proliferation.

12. The method of claim 11 in which the monospecific anti-cyclin A antibody binds to the peptide KYHGVSLLNPPETLNL, termed P1.

13. The method of claim 11 in which the monospecific anti-cyclin A antibody binds to the peptide CLVEVGEEYKLQNE, termed P2.

14. The method of claim 11 in which the monospecific anti-cyclin A antibody binds to the peptide CIHVDEAEKEAQKKPAE, termed P3.

15. The method of claim 11 in which the monospecific anti-cyclin A antibody binds to the peptide CLMDLHQTYLKAPQHAQQSIREKYKNSKYHG, termed P4.

16. The method of claim 11 in which the disorder of cellular proliferation is malignancy.

17. The method of claim 12 in which the disorder of cellular proliferation is malignancy.

18. The method of claim 13 in which the disorder of cellular proliferation is malignancy.

19. The method of claim 14 in which the disorder of cellular proliferation is malignancy.

20. The method of claim 15 in which the disorder of cellular proliferation is malignancy.

21. A method of treating a disorder of cellular proliferation comprising administering, to a patient in need of such treatment, an effective amount of monospecific anti-cyclin A antibody, such that actively dividing cells bearing PDPK may be damaged and/or killed.

22. The method of claim 21 in which the monospecific anti-cyclin A antibody binds to the peptide KYHGVSLLNPPETLNL, termed P1.

23. The method of claim 21 in which the monospecific anti-cyclin A antibody binds to the peptide CLVEVGEEYKLQNE, termed P2.

24. The method of claim 21 in which the monospecific anti-cyclin A antibody binds to the peptide CIHVDEAEKEAQKKPAE, termed P3.

25. The method of claim 21 in which the monospecific anti-cyclin A antibody binds to the peptide CLMDLHQTYLKAPQHAQQSIREKYKNSKYHG, termed P4.

26. The method of claim 21 in which the disorder of cellular proliferation is malignancy.

27. The method of claim 22 in which the disorder of cellular proliferation is malignancy.

28. The method of claim 23 in which the disorder of cellular proliferation is malignancy.

29. The method of claim 24 in which the disorder of cellular proliferation is malignancy.

30. The method of claim 25 in which the disorder of cellular proliferation is malignancy.

31. A monoclonal antibody that binds to P3 and that competitively inhibits the binding of monoclonal antibody CHLA-3, as produced by hybridoma CHLA-3-13 that is deposited with the American Type Culture Collection and assigned accession number _____, to P3.

32. A monoclonal antibody produced by hybridoma CHLA-3-13, deposited with the American Type Culture Collection and assigned accession number _____.

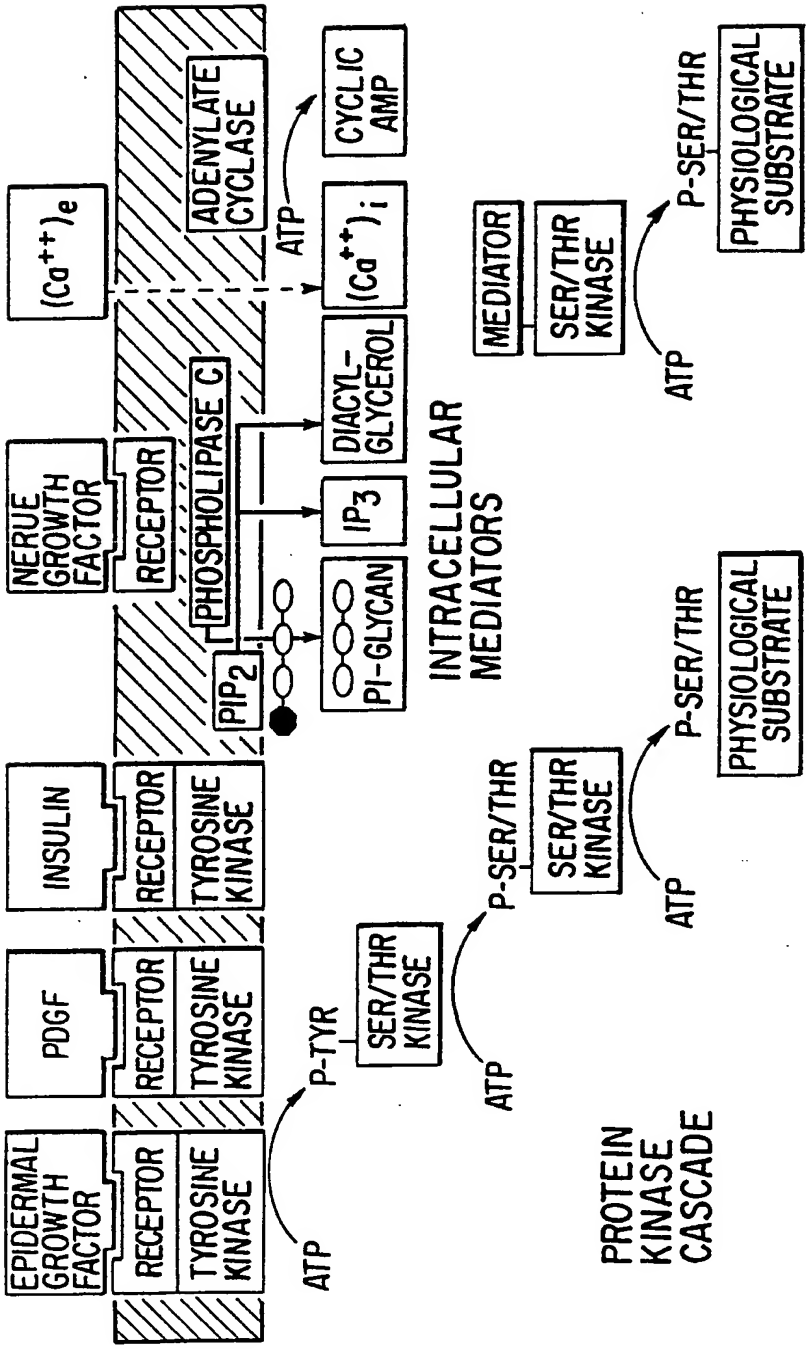
EPIDERMAL GROWTH FACTOR
RECEPTOR
TYROSINE KINASE
PDGF
RECEPTOR
TYROSINE KINASE
INSULIN
RECEPTOR
TYROSINE KINASE
NERUE GROWTH FACTOR
RECEPTOR
PHOSPHOLIPASE C
ADENYLATE CYCLASE
$(Ca^{++})_e$
ATP
P-TYR
SER/THR KINASE
ATP
P-SER/THR
SER/THR KINASE
ATP
P-SER/THR
PHYSIOLOGICAL SUBSTRATE
PROTEIN KINASE CASCADE
PIP2
PI-GLYCAN
IP3
DIACYL-GLYCEROL
$(Ca^{++})_i$
CYCLIC AMP
INTRACELLULAR MEDIATORS
MEDIATOR
SER/THR KINASE
ATP
P-SER/THR
PHYSIOLOGICAL SUBSTRATE

FIG. 1

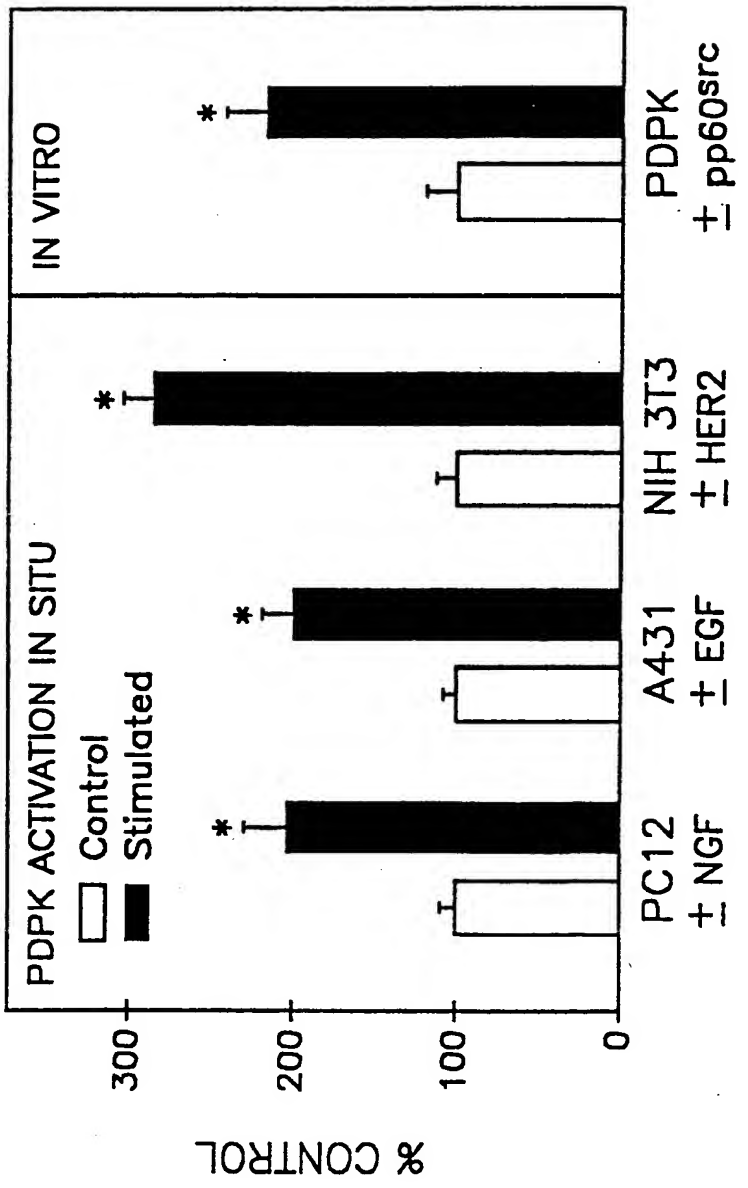
PDPK ACTIVATION IN SITU
Control
Stimulated
IN VITRO
% CONTROL
300
200
100
0
PC12
± NGF
A431
± EGF
NIH 3T3
± HER2
PDPK
± pp60src

FIG. 2

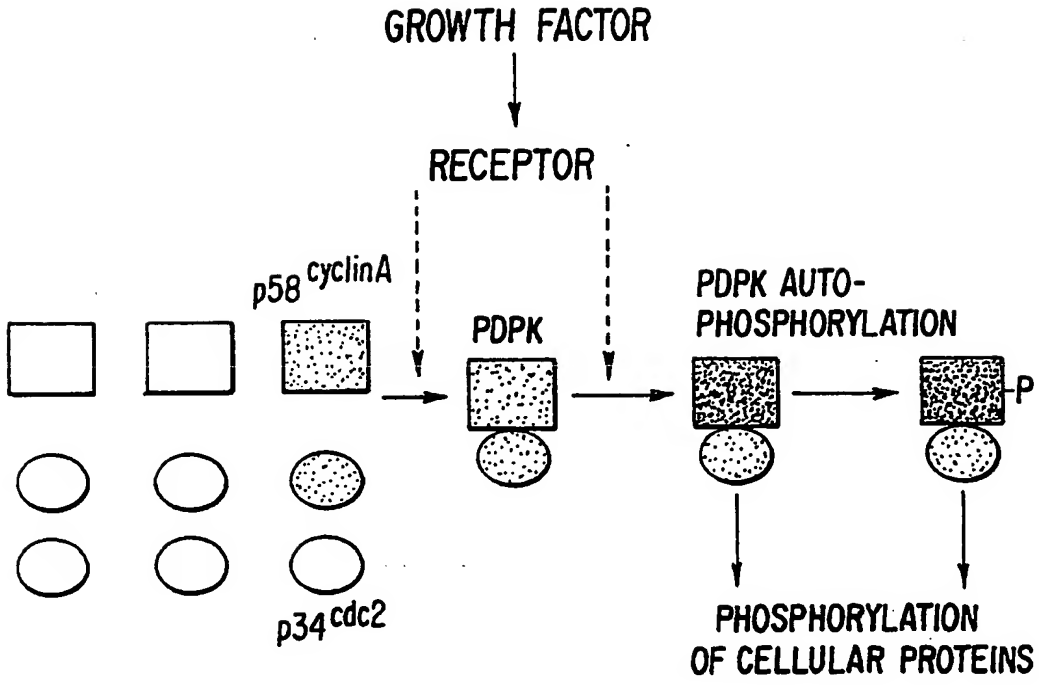
GROWTH FACTOR
RECEPTOR
p58 cyclinA
PDPK
PDPK AUTO-
PHOSPHORYLATION
P
p34 cdc2
PHOSPHORYLATION
OF CELLULAR PROTEINS

FIG. 3

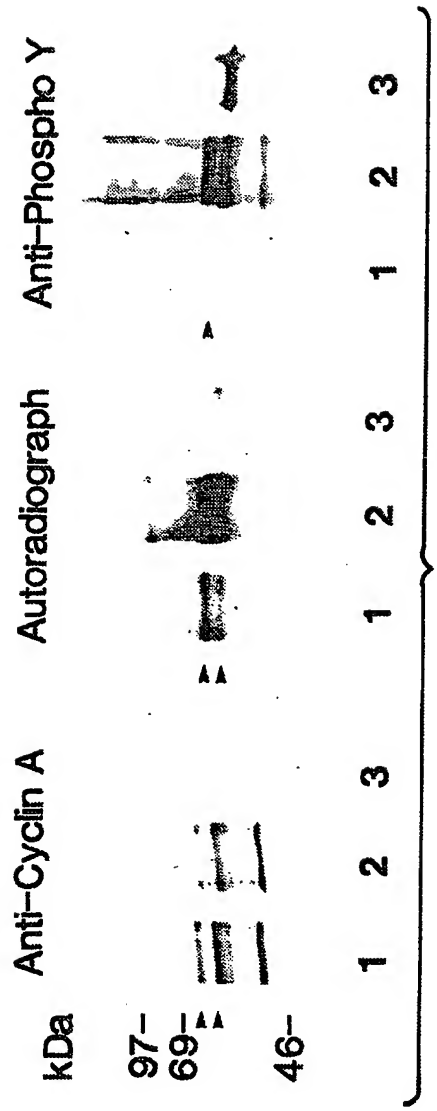
kDa
97–
69–
46–
Anti–Cyclin A
1 2 3
Autoradiograph
1 2 3
Anti–Phospho Y
1 2 3

FIG. 4

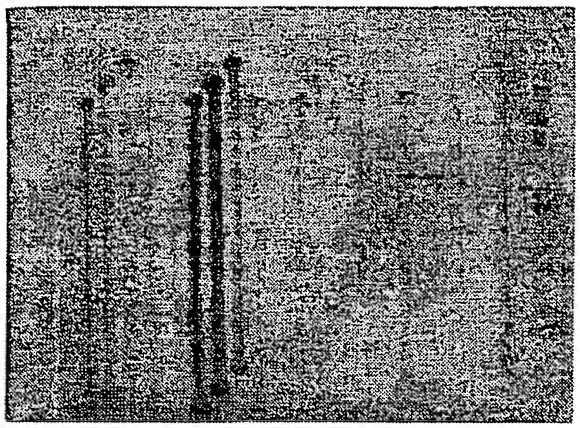

FIG. 5A

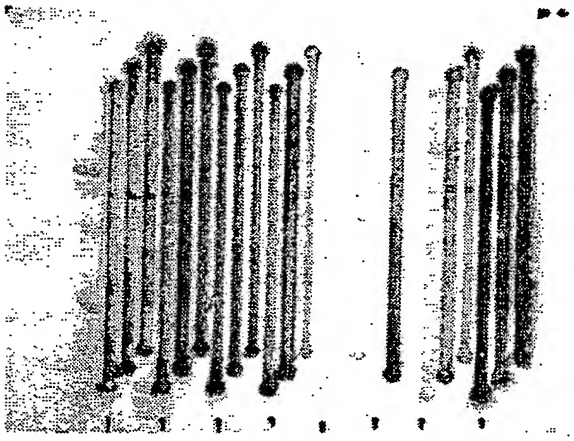

FIG. 5B

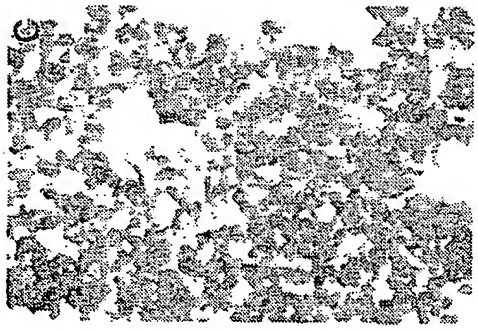

FIG. 6C

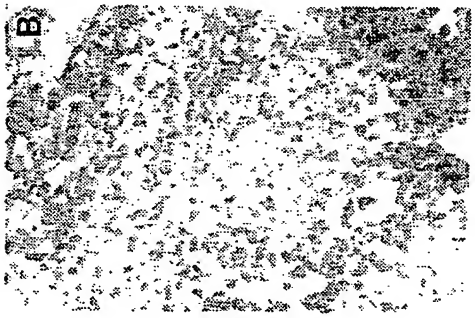

FIG. 6B

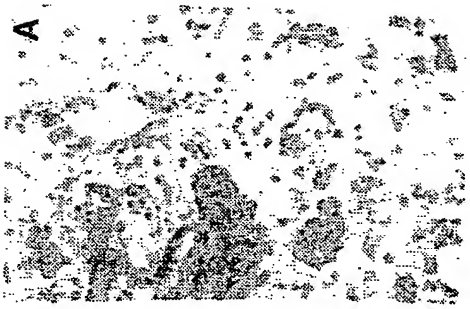

FIG. 6A

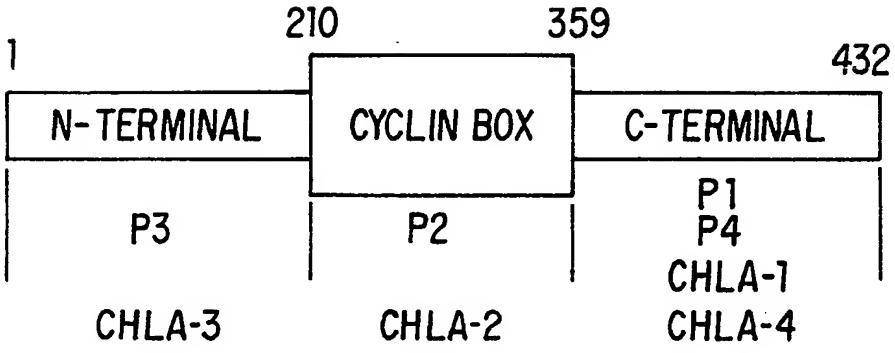
1
210
359
432
N-TERMINAL
CYCLIN BOX
C-TERMINAL
P3
P2
P1
P4
CHLA-1
CHLA-3
CHLA-2
CHLA-4

FIG. 7A

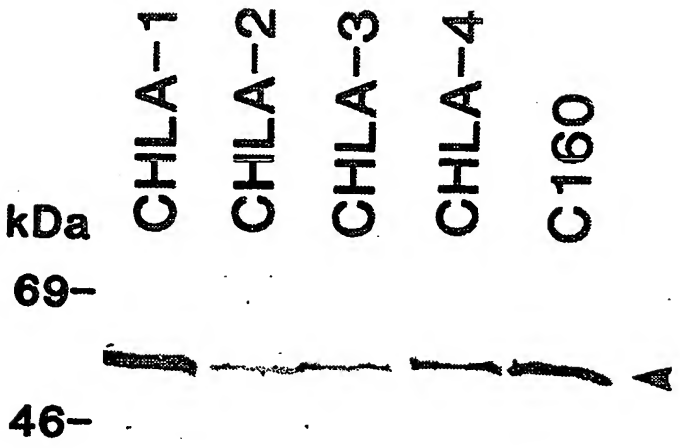

FIG. 7B

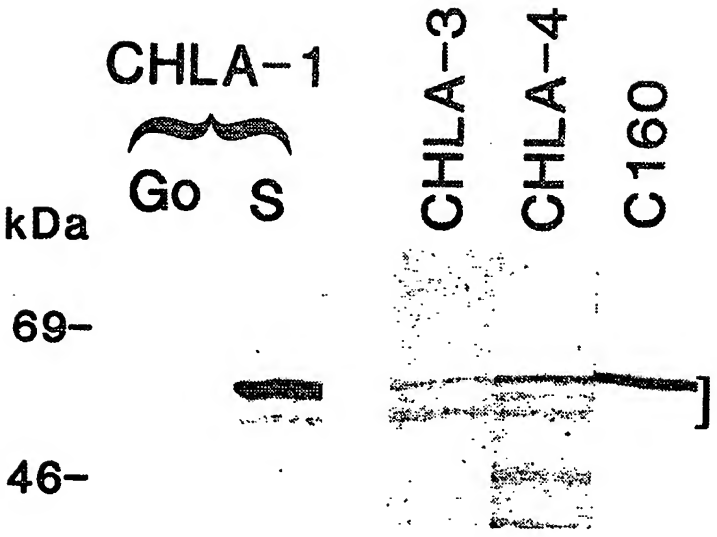

FIG. 7C

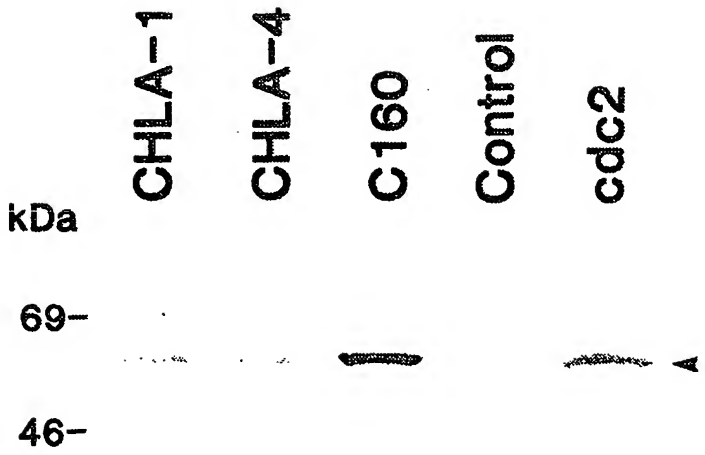

FIG. 7D

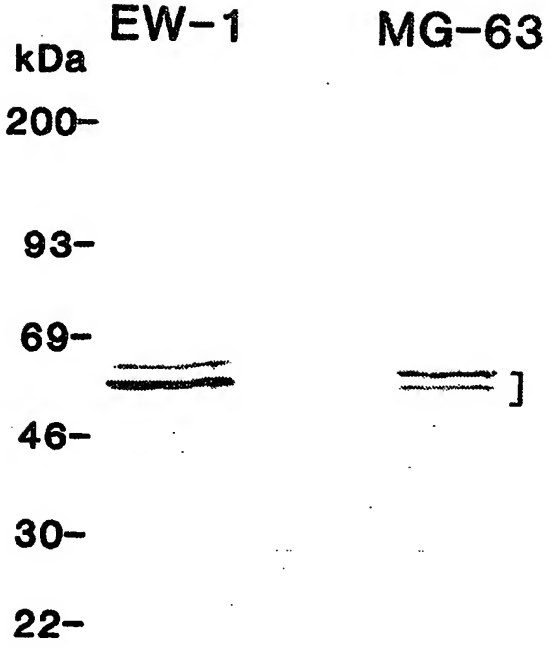

FIG. 7E

INTERNATIONAL SEARCH REPORT

In ational application No.
PCT/US92/03696

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/02, 39/00; C07K 7/00, 13/00; G01N 33/53, 33/536, 33/541
US CL :424/85.8; 436/501, 536, 540, 547, 548; 530/300, 387, 388
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85.8

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG (BIOSCIENCE), APS, INTELLIGENETICS, GEN-SEQ, SWISS-PROT 21, PIR (SEQUENCE DATA BASES)
SEARCH TERMS: FREDERICK L. HALL, CYCLIN A

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CELL, Volume 58, issued 08 September 1989, A. Giordano et al., "A 60 kD cdc2-Associated Polypeptide Complexes with the E1A Proteins in Adenovirus-Infected Cells", pages 981-990, see entire document.	1-10, 31-32
Y	J.BIOL. CHEM., Volume 264, Number 27, issued 25 September 1989, P.R. Vuillet, "Identification of a Novel Proline-Directed Serine/Threonine Protein Kinase in Rat Pheochromocytoma", pages 16292-16298, see entire document, particularly Table 1.	1-10, 31-32
Y	J. BIOL. CHEM., Volume 265, Number 12, issued 25 April, 1990, F.L. Hall, "Phosphorylation of Synapsin I at a Novel Site by Proline-Directed Protein Kinase", pages 6944-6948, see entire document, particularly Figure 6.	1-10, 31-32
Y	NATURE, Volume 343, issued 08 February 1990, J. Wang et al., "Hepatitis B Virus Integration in a Cyclin A Gene in a Hepatocellular Carcinoma", pages 555-557, see entire document, particularly Figures 2 and 3.	1-32

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
28 JULY 1992	05 AUG 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer PHILLIP GAMBEL
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

Int ational application No.
PCT/US92/03696

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NATURE, Volume 346, issued 23 August 1990, J. Pines et al., "Human Cyclin A is Adenovirus E1A-Associated Protein p60 and Behaves Differently from Cyclin B, pages 760-763, see entire document.	1-10, 31-32
Y,P	J. BIOL. CHEM., Volume 266, Number 26, issued 15 September 1991, F.L. Hall et al., "Characterization of the Cytoplasmic Proline-Directed Protein Kinase in Proliferative Cells and Tissues as a Heterodimer Comprised of $p34^{cdc2}$ and $p58^{cyclin\ A}$, pages 17430-17440, see entire document, particularly Table 1.	1-32